

Monitoring of Brewing Yeast Propagation Under Aerobic and Anaerobic Conditions Employing Flow Cytometry

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ABSTRACT

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The vitality and viability of industrial strains of *Saccharomyces cerevisiae* was monitored during pilot plant experiments simulating yeast propagation under aerobic and anaerobic conditions. Industrial wort of 12°P original gravity was used as a growth substrate for yeast propagation. The work was carried out with three widely used Czech lager yeast industrial strains: strains 2, 7 and 95. Cell cycle, cell size, granularity, glycogen content, DNA and protein content were analyzed by flow cytometry. Significantly higher specific growth rates, higher content of yeast glycogen, earlier G2/M phase cells maximum, and faster cell protein creation was observed under aerobic conditions compared to anaerobic. Strains 7 and 95 showed losses in flocculation ability after aerobic propagation compared to anaerobic propagation. Under either aerobic or strictly anaerobic conditions, only strain 2 did not show a significant loss in flocculation ability.

Key words: Cell cycle, fluorescent methods, proteins, yeast glycogen, yeast propagation.

INTRODUCTION

Fresh yeast in optimal physiological state is one of the most important ingredients for favorable, successful and reproducible fermentations²¹. In recent years attention has been focused on finding new ways to improve yeast quality control and yeast management procedures. Brewing yeasts are traditionally propagated through a series of poorly aerated batch fermentation vessels with low biomass yield and relatively long times of cultivation¹⁶. Some authors^{6,17,20} have described how under aerobic conditions a significantly increased biomass yield with an improved physiological state could be obtained. Nevertheless aerobic propagation incorporates some challenges that have to be overcome, such as maintaining optimal oxygen saturation, controlling foam formation and monitoring of changes of yeast behavior (e.g., related with substrate consumption and metabolite production).

Harrison et al.⁹ also observed changes in yeast cell surface hydrophobicity, surface charge and in flocculation ability. Alteration of flocculation properties generally causes profound changes in the settling behavior of yeast cells and this is an important measurable parameter of industrial yeast strains⁸. Sedimentation remains the basis for most of the modern flocculation assays, but the rate at which a flocculent yeast suspension sediments is a function of the floc size, cell concentration, floc density, and the extent of agitation imposed on the yeast suspension prior to the settling period⁷.

Many authors^{12,13,18} have described the use of a precise, quick and reproducible fluorescent optical method, flow cytometry, for monitoring physiological changes in *Saccharomyces cerevisiae*. Flow cytometric measurement allows for the analysis of single yeast cells and measurement speeds of up to 3000 cells per second are possible, however recommended measurement speeds are generally lower. In principle, the yeast cells in suspension are passing a flow channel in a narrow stream. A laser beam is directed onto the flow channel. The yeast cells pass the analysis point in which they are illuminated. The light is scattered into different directions depending on the refractive index, size and shape of each cell. The cell size is indicated by light scattered in small angles of the laser beam (forward scatter, FSC). Scattered light, which is collected in a cone in the orthogonal direction of the laser beam, is called side scatter (SSC). This parameter also indicates size, but is influenced mainly by the surface and internal structures of the cells. Fluorescence optical analysis employs fluorescent probes that are highly specific for components on the surface membranes or within the cells. These probes, attached to the cell component of interest, are excited by laser light of a wavelength specific for the particular fluorescent stain used. According to Stokes Law, the fluorescent dye emits light with a wavelength that is longer than the wavelength of the excitation light. The obtained distribution and/or intensity of these emitted fluorescence signals indicate various states and functionality depending on the staining mechanism. The emission fluorescence signals are detected by several fluorescence detectors and they can be recorded digitally and quantified by a specially developed software program¹⁸ (Fig. 1).

During propagation, yeast cells produce the storage carbohydrate polymer glycogen, which is accumulated in the latter stages of the fermentation²³. Changes in the glycogen content of yeast cells, under aerobic and anaerobic conditions, were investigated in the course of this study. A

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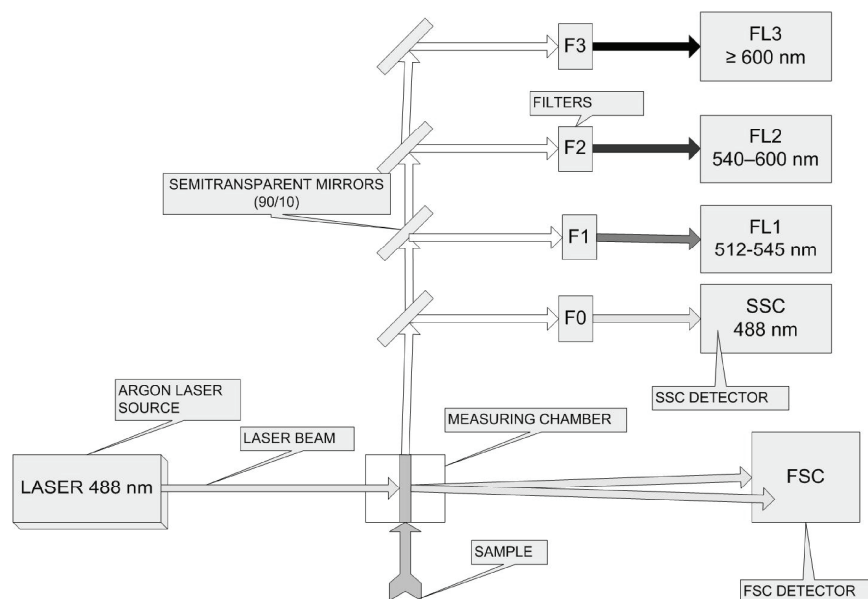


Fig. 1. Basic principles of flow cytometry.

rapid method for flow cytometric determination of glycogen and cell protein in yeast cells has been developed by Hutter^{12,13}.

The measurement of the DNA content of yeast cells was one of the first major applications of flow cytometry in brewing and is still one of the main applications of this method in this field. The DNA content of yeast cells provides information about the cell cycle and is thus a powerful tool to assess the effects of propagation parameters on the yeast cell cycle and yeast physiology. A large number of fluorescent dyes specific for DNA were developed, most of which can be adapted easily for staining of yeast DNA and cell cycle evaluation. The most common DNA dyes are Hoechst 33342, DAPI, propidium iodide and TO-PRO-3. Generally, the staining procedure involves cells being fixed in ethanol in order to make the yeast cell wall permeable for the DNA fluorochromes. RNA is also stained by the DNA dyes and thus interferes during DNA determination and is removed by RNAase digestion¹⁸.

MATERIALS AND METHODS

Three proprietary bottom fermenting Czech yeast strains, from a culture collection of brewing yeasts, internationally registered under the code name RIBM 655 (RIBM – Research Institute of Brewing and Malting) as strains numbers 2, 7 and 95, were utilized in this study. Stock cultures of the strains were preserved on YPD agar slopes.

Table I. Composition of industrial wort.

	Content
Zn	0.15 mg/L
Ca	50 mg/L
Mg	100 mg/L
Bitterness units	37 B.U.
Extract	13.8°P

Specific growth rates were calculated from the curve of the biomass increase during the time of propagation. Samples for measurement were taken every two hours. The specific growth rate was determined as the slope of the log value of biomass concentration vs. time plot.

All media were sterilized by autoclaving for 21 min at 121°C immediately after preparation. The yeast starter cultures for use in the bioreactor were grown aerobically for 36 h at 25°C with shaking at 150 rpm in an Erlenmeyer flask containing 600 mL of industrial brewing wort 12°P (Table I). One colony from the agar slope was directly immersed under aseptic conditions in the 600 mL of 12°P industrial brewing wort and used as starting amount of yeast for the first step.

Industrial brewing wort with a gravity of 13.8°P was diluted with pre-sterilized tap water to achieve 12°P wort (Table I). To prevent excessive foaming a drop of Anti-foam 204 (Sigma Aldrich) was added to the wort. The temperature was maintained at 12°C during the yeast propagation. Prior to pitching the wort was aerated till maximum oxygen saturation (monitored with a pO₂ probe) was reached. During aerobic propagation, the oxygen concentration was also monitored and it changed from 100% to 5%; once the level of 5% was reached the aeration intensity was increased in order to keep the dissolved oxygen above 5% during this stage.

Experiments were performed in a 5 L laboratory bioreactor (B. Braun Biotech International, Melsungen, Germany owned by Sartorius) with a cooling jacket and with controlled temperature, agitation, pH, and pO₂ level (this last parameter was controlled with the aid of a mass flow controller (Hastings, U.S.A.) which would increase or decrease the gas flow rate as needed to keep pO₂ at the desired level. The experiments were performed under aerobic and anaerobic conditions. In aerobic experiments the oxygen level was maintained above 5% of maximum saturation as described previously. In anaerobic experiments, the wort was aerated only once prior to pitching

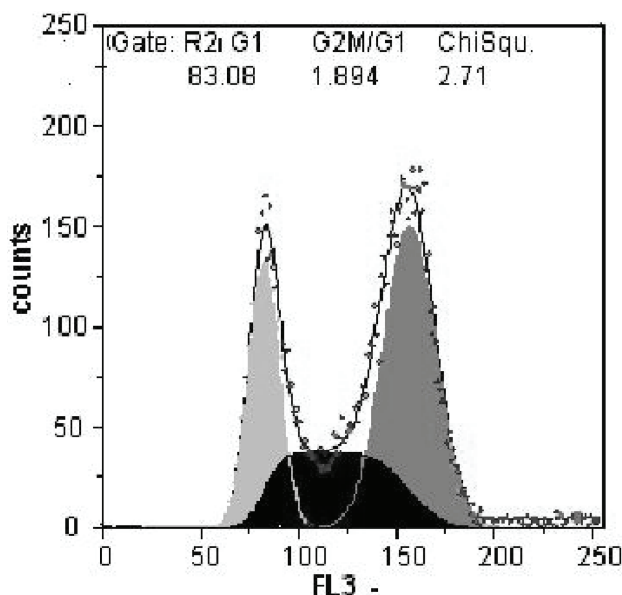


Fig. 2. Cell cycle analysis based on the function “Cell cycle analyses” from the PAS III FloMax software menu.

and the fermentation was performed without aeration. The yeast flocculation ability was examined with the modified Stratford flocculation test and the pre-controlled micro sedimentation test¹⁹.

Flow-cytometric analysis was performed with a PAS III from Partec (Münster, Germany)²². An argon ion laser with a light wavelength of 488 nm was used for the excitation of the fluorescent dyes.

The amount of yeast cells used for the staining procedures varied between 20 and 60 million cells per mL. The yeast cell cycle was monitored using the procedure described below. The fixed yeast cells were incubated in 0.1% RNA-ase solution for 40 min at 37°C.

After removal of the RNA-ase solution by centrifugation, the yeast pellet was re-suspended in phosphate buffered saline (PBS) solution. After this the yeast cells were spun down in a centrifuge and the supernatant was gently removed. A 1 mL of propidium iodide staining solution (50 µg/mL) was added to the cell pellet and the cells were re-suspended. The cells were put into contact with the staining solution for 40 min in the dark. After the staining procedure yeasts were harvested by centrifugation and washed twice in PBS solution to avoid background con-

tamination by the fluorescent dye. The now fluorochromised cells were subjected to flow-cytometric analysis at an excitation wavelength of 488 nm; the fluorescent emission was detected by a fluorescence detector FL3 (wavelength detected ≥ 600 nm). The histogram of fluorescent intensity was evaluated using the function “Cell cycle analyses” from the PAS III FloMax software menu. The distribution of yeasts cells in the different cell cycle stages was provided by this function^{11,15} (Fig. 2). These measurements were repeated three times in every measurement point and averaged value was used as result.

Prior to the measurement of glycogen and protein content, an optimization procedure for fluorescent dye concentration to be used was carried out and the graduating fluorescent dye solutions were prepared and used for the staining procedure. In case of acriflavine (ACR) it was 0.001, 0.01, 0.1, 0.5 and 1 mg/mL and in the case of fluorescein isothiocyanate (FITC) it was 0.0001, 0.0005, 0.001, 0.01 and 0.1 mg/mL. For each value, the mean value of fluorescent intensity was measured by flow cytometry and the optimal dye concentration was determined. The optimal concentration of dye was further used for yeast staining. Measurements were repeated ten times and measure error was calculated.

Glycogen content was measured according to Hutter et al.¹⁰ Yeast cells were retrieved from the propagation culture and were washed twice in PBS buffer and incubated in 1 mL of 1 mol/L solution of HCl for 50 min. HCl was removed by centrifugation and 10 µL of the 0.1 mg/mL acriflavine hydrochloride solution (Sigma Aldrich A8251, emission max. 512 nm) was added and the cells were incubated for 20 min at room temperature in the dark. After the staining procedure yeast were harvested by centrifugation and washed twice in PBS solution.

The fluorochromised cells were subjected to flow cytometric analysis using the 488 nm excitation wavelength and emission was detected by FL1 detector (detection from 512 nm to 545 nm). These measurements were repeated three times in every measurement point and averaged value was used as result.

The protein content of single yeast cells was determined employing the fluorescent probe fluorescein isothiocyanate (FITC, Sigma Aldrich, F-1628, emission max. 516 nm to 525 nm). After washing and spinning down the yeast cells, 100 µL PBS buffer and 10 µL FITC solution (1 µg/mL of acetone) were added. Samples were incubated at 0°C for 30 min and immediately analyzed by

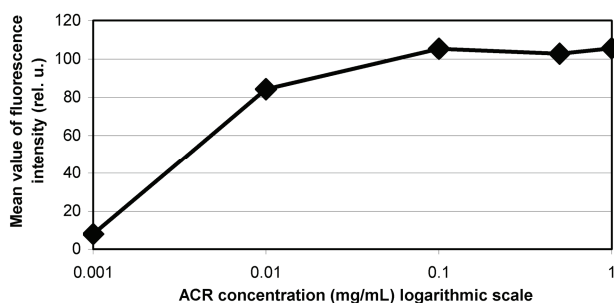


Fig. 3. Optimization of the staining procedure with acriflavine (ACR).

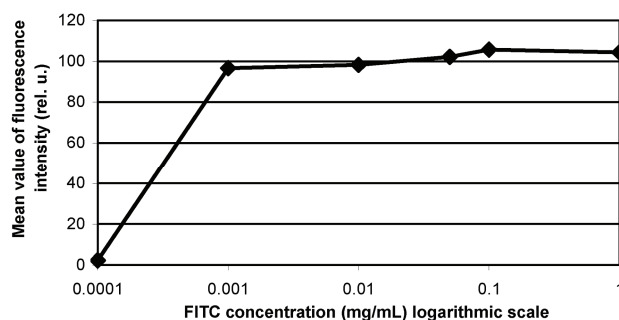


Fig. 4. Optimization of the staining procedure with fluorescein isothiocyanate (FITC).

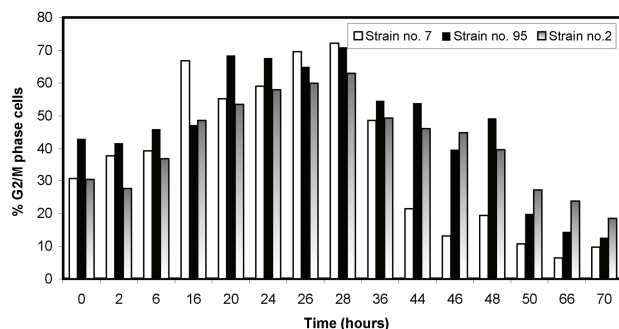


Fig. 5. Changes in proportion of G2/M phase in yeast strains 2, 7 and 95 during anaerobic propagation.

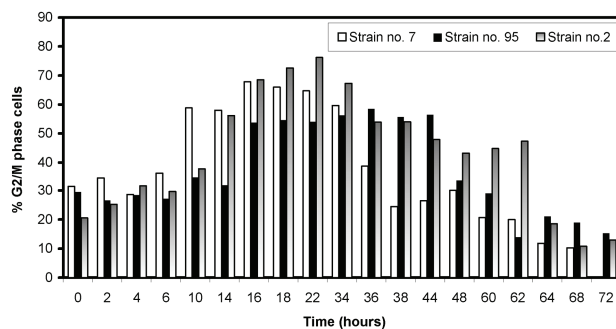


Fig. 6. Changes in proportion of G2/M phase in yeast strains 2, 7 and 95 during aerobic propagation

flow cytometry, (excitation at 488 nm and emission was detected at 512 nm to 545 nm with the fluorescent detector FL1). These measurements were repeated three times in every measurement point and averaged value was used as result.

RESULTS AND DISCUSSION

Optimization of the staining procedures for flow cytometric analysis of glycogen and protein content in yeast cells has shown that an acriflavine concentration of 0.1 mg/mL is optimal and should produce the most accurate results for the glycogen determination (Fig. 3). The optimal concentration of FITC for the determination of yeast protein content was identified as being 0.001 mg/mL (Fig. 4). The measure error determined from ten repetitions was for acriflavine 14% and for fluorescein isothiocyanate 15%.

The specific growth rate for strain 2 was 0.29 h^{-1} , for strain 95 was 0.28 h^{-1} and for strain 7 was 0.26 h^{-1} . There were no significant differences between the three tested yeast strains with regard to specific growth rates.

In wort, as expected, specific growth rates, measured under aerobic and anaerobic conditions, were notably different between the three yeast strains tested. Specific growth rates of all tested strains were, approximately three times higher under aerobic conditions, compared to the anaerobic system (Table II). The observed increase in specific growth rate values is in accordance with the results described in several works^{4,5,9,14}.

The cell cycle of the three yeast strains was evaluated by flow cytometric measurement during both the aerobic and anaerobic propagation experiments. This evaluation concentrated on the proportion of yeast cells in the G2/M cell cycle phase in the industrial strain populations. Pitching yeasts from the shaking flasks contained between 20 and 40% of G2/M phase cells. This relatively high con-

centration of G2/M phase cells was most probably caused by the use of exponentially growing cultures from the shake flasks. Observed amount of G2/M phase cells in exponentially growing culture was generally in accordance with findings of Hutter et al.^{10,11} The first hours of both, aerobic and anaerobic propagations were characterized by an increase of G2/M phase cells in the population (Figs. 5 and 6). The pattern of increase of G2/M phase cells has shown differences between the tested yeast strains and also between the aerobic and anaerobic propagations. Strain 2 displayed the slowest increase of G2/M phase cells and was the last to reach the maximum proportion of G2/M phase cells. Strain 7 has reached the maximum concentration of G2/M phase cells in 16 h of aerobic propagation and in 26 h in the case of anaerobic propagation. Strain 95 reached the maximum concentration of G2/M phase cells between 16 and 44 h of aerobic propagation and in 28 h in the case of anaerobic propagation. Strain 2 reached the maximum concentration of G2/M phase cells in 22 h of aerobic propagation and in 28 h in the case of anaerobic propagation. By comparing the anaerobic propagation system (Fig. 5) with the aerobic system (Fig. 6), it can be seen that the maximum concentration of G2/M phase cells was achieved faster under aerobic conditions. It is known²¹ that cells in G2/M phase are very close to cell dividing and to return back to the G1 phase where the metabolism is most intensive and therefore some publications^{10,13} describe the maximum concentration of G2/M phase yeast cells in a pitching yeast culture as the optimum condition for pitching into industrial brewing wort. The above mentioned facts and results show that the optimal time for pitching propagated yeast cultures is different not only for each yeast strain, but also depends on the aeration regime during propagation.

Glycogen content was measured according to Hutter et al.¹⁰ with minor modifications as outlined in the Material and Methods chapter. Glycogen content in the yeast cells varied during the propagation experiments and the curves describing yeast glycogen content had a similar shape for all yeast strains and aeration regimes (Figs. 7 and 8). The fermentations at 20 h were characterized by the rapid depletion of the yeast glycogen content. After reaching the lowest level of concentration, glycogen content did not change significantly for approximately 20 h of fermentation. All tested yeast strains exhibited slow glycogen accumulation during the last phase of fermentation (between 40 and 70 h of fermentation). The tested strains showed

Table II. Specific growth rates of strains 2, 7 and 95 under aerobic and anaerobic conditions.

Anaerobic system		Aerobic system	
Strain	Specific growth rate (h^{-1})	Strain	Specific growth rate (h^{-1})
2	0.019	2	0.061
7	0.016	7	0.057
95	0.020	95	0.052

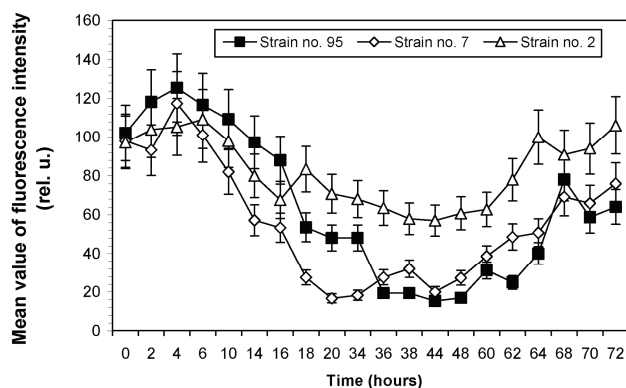


Fig. 7. Glycogen content in yeast strains 2, 7 and 95 during anaerobic propagation.

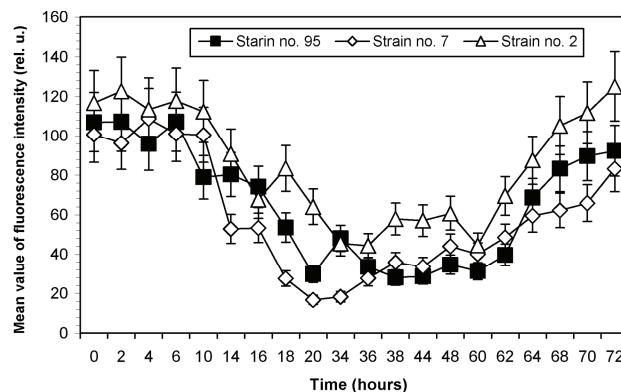


Fig. 8. Glycogen content in yeast strains 2, 7 and 95 during aerobic propagation.

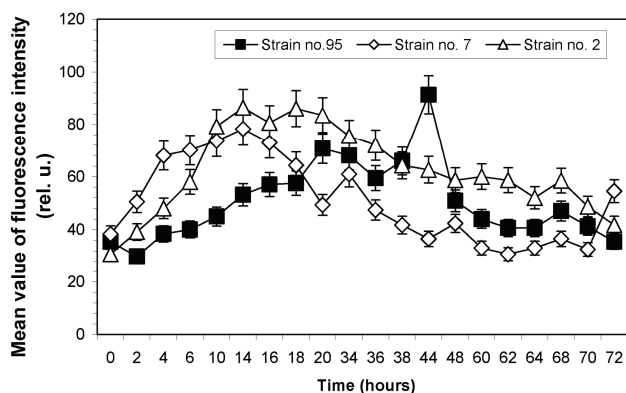


Fig. 9. Protein content in yeast strains 2, 7 and 95 during anaerobic propagation.

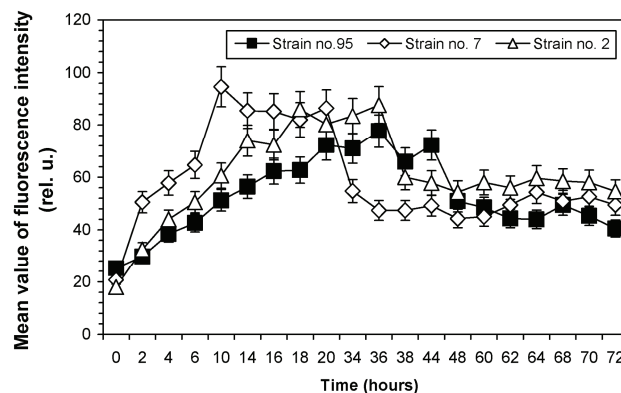


Fig. 10. Protein content in yeast strains 2, 7 and 95 during aerobic propagation.

different tendencies to consume and accumulate glycogen. The most rapid glycogen depletion was observed with strain 7, slowest by strain no. 2 and no. 95. Strain no. 7 is known as a strain with a short lag phase and the highest specific growth rate at the beginning of fermentation³, this being in agreement with the work of Hutter et al.¹⁰ that showed that speed of storage carbohydrate (glycogen and trehalose) depletion correlated well with specific growth rates of the strains. Glycogen level in the yeast is most probably related to the activity of glycolysis and of the enzymes of carbohydrate metabolism. Basarova et al.³ showed that strain no. 7 also had the fastest depletion of glucose, fructose and maltose and the fastest decrease of activity represented by specific growth rate, results that correlated well with glycogen curves shown in this work. Strain 2 displayed the highest glycogen accumulation potential in comparison to the other strains. The higher potential for glycogen accumulation by strain no. 2 was observed by Basarova and Blaha³ too. By comparison of the aerobic and anaerobic propagation, it was observed that glycogen curves (Figs. 7 and 8) are very similar. Nevertheless, aeration during propagation leads, in case of strains no. 2 and no. 95, to higher glycogen accumulation in the later phases of fermentation and in case of aerobic propagation the faster rate of glycogen increase was also observed. Hutter et al.¹⁰ did not observe an influence of aeration on glycogen accumulation in the later phases of fermentation. Such a contradiction could be partly ex-

plained by different strains and conditions used during the propagation.

The protein content was measured in accordance with the procedure described in the Material and Methods chapter. The content of proteins in the yeast cell varied notably during the propagation experiments and the curves describing yeast protein content had similar tendencies for all yeast strains and aeration conditions (Figs. 9 and 10). The first hours of fermentation were characterized by an increase in protein content of the cells. This trend can be interpreted as an increase of enzymatic activity within the cell in the earlier phases of fermentation as was described in several works^{1,21}. In work of Alberghina¹, it was also shown that higher protein content correlates with the yeast growth rate which is linked with enzymatic activity²¹. The fastest increase in cell protein at the beginning of fermentations was found with strain no. 7. As was described in the previous paragraph, strain no. 7 has, among the studied strains, the shortest lag phase and fastest specific growth rate at the beginning of fermentation and therefore is expected to have a higher enzymatic activity in this part of propagation step as compared to the others. As a consequence of the observed growth rates, a significant decrease in yeast protein content of strain no. 7 occurred between 36 and 44 hours of propagation compared to strains no. 2 and 95 where a significant decrease was observed between 38 and 60 hours. A faster increase in cell protein levels and an earlier start of protein de-

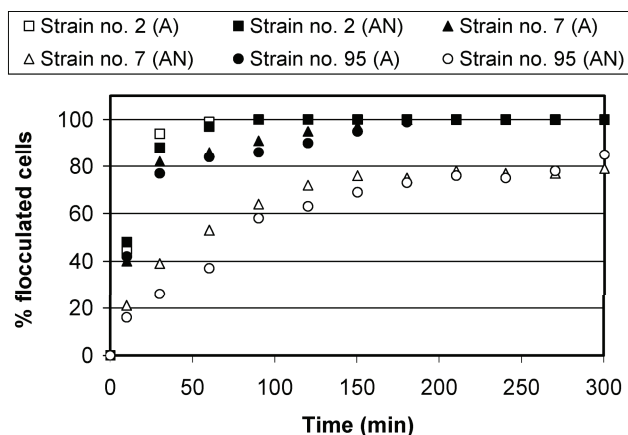


Fig. 11. Percentage of flocculated cells during aerobic and anaerobic propagation (A, aerobic propagation, AN, anaerobic propagation).

crease compared to anaerobic propagation was observed during aerobic propagation (Figs. 9 and 10). This result can be explained by higher specific growth rates and by higher yeast activity (metabolic, growth etc) under aerobic conditions.

The flocculation potential of the tested strains was determined after 72 h of propagation. These measurements focused mainly on determining the influence of aeration on the flocculation ability of the yeast strains. Comparing aerobic and anaerobic propagation, it was detected that strains 7 and 95 were affected and showed a decrease in flocculation ability after aerobic propagation and on the other hand, strain 2 did not show any loss of flocculation ability after the aerobic propagation (Fig. 11). Flocculation phenomena are known²¹ to be related to the composition of yeast surface proteins and with other surface properties of yeasts^{9,21}. Harrison et al.⁹ describe the influence of aeration on hydrophobicity and flocculation of flocculent and non flocculent strains, showing that aeration leads to increasing flocculation ability of flocculent strains and decreasing in the case of non flocculent strains. Strain no. 2 is described as a highly flocculent strain, and strains no. 7 and no. 95 as a low or medium flocculent strains (Basarova et al.³). In accordance with what was concluded by Harrison, the flocculation ability of the highly flocculent strain was unchanged or slightly improved and flocculation ability of strains described as low or medium flocculent were affected by aeration. In our case, although characterized as flocculent, tested strains showed different responses to aeration. Obtained results indicated that aeration can have a negative effect on the flocculation characteristics of flocculent strains and that the characterization of this effect requires a precise characterization of strain flocculation. So far, no general conclusions about the influence of aeration on flocculation ability can be obtained, although it is clear that it is strain dependent.

CONCLUSIONS

The following can be concluded based on the results presented:

Optimal acriflavine concentration for the staining of yeast glycogen content was determined to be 0.1 mg/mL.

Optimal FITC concentration for staining of yeast cell proteins was 0.001 mg/mL.

The biomass growth rates and yields of all strains were improved on aeration during the propagation stage, as expected.

The higher glycogen accumulation and faster rate of glycogen increase in the later phases of fermentation was observed in case of the aerobic propagation of strains no. 2 and no. 95.

The fastest protein increase during propagation was measured for strain 7. This correlates with the fastest glycogen depletion and sugar consumption observed for this strain

Flocculation potential of strains 7 and 95 was affected by aeration during yeast propagation. It appears that the flocculation ability of industrial strains depends mainly on the genetic properties of each strain, however some could be affected by aeration.

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